US Environmental Protection Agency
Office of Pesticide Programs

Office of Pesticide Programs
Microbiology Laboratory
Environmental Science Center, Ft. Meade, MD

Standard Operating Procedure for
Disinfectant Towelette Test:
Testing of Mycobacterium bovis (BCG)

SOP Number: MB-23-02

Date Revised: 03-05-13

SOP Number	MB-23-02
Title	Disinfectant Towelette Test: Testing of *Mycobacterium bovis* (BCG)
Scope	Describes the methodology used to determine the efficacy of towelette-based disinfectants against *Mycobacterium bovis* (BCG) on hard surfaces. The test is based on AOAC Method 961.02 (Germicidal Spray Products as Disinfectants). See 15.1.
Application	For product evaluations under the Antimicrobial Testing Program (ATP), a study protocol is developed which identifies the specific test conditions for a product sample such as contact time, neutralizers, etc.

	Approval Date
SOP Developer:	_____ Print Name: _____
SOP Reviewer	_____ Print Name: _____
Quality Assurance Unit	_____ Print Name: _____
Branch Chief	_____ Print Name: _____

Date SOP issued:	
Controlled copy number:	
Date SOP withdrawn:	

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1. **Definitions**	Additional abbreviations/definitions are provided in the text. Carrier Set = One "carrier set" is defined as the primary MPB tube containing the carrier and duplicate tubes of the two additional subculture media (e.g., M7H9 broth, Kirchners medium, or TB broth) inoculated from the carrier's corresponding neutralizer tube for a total of 5 tubes per carrier. There are 10 carrier sets per disinfectant tested.
2. **Health and Safety**	Follow procedures specified in SOP MB-01, Laboratory Biosafety. The Study Director and/or lead analyst should consult the Material Safety Data Sheet for specific hazards associated with products.
3. **Personnel Qualifications and Training**	Refer to SOP ADM-04, OPP Microbiology Laboratory Training.
4. **Instrument Calibration**	Refer to SOP EQ-01, EQ-02, EQ-03, EQ-04 and EQ-05 for details on method and frequency of calibration.
5. **Sample Handling and Storage**	Refer to SOP MB-22, Disinfectant Sample Preparation, and SOP COC-01, Chain of Custody Procedures.
6. **Quality Control**	For quality control purposes, the required information is documented on the appropriate form(s) (see section 14).
7. **Interferences**	1. The carriers inside the Petri dishes should be dry prior to inoculation. Moisture can interfere with the concentration and drying of the inoculum on the glass slide carrier. 2. Any inoculated carrier that is wet at the conclusion of the carrier drying period should not be used.
8. **Non-conforming Data**	1. Sterility and/or viability controls do not yield expected results. 2. The mean log density for control carriers falls outside the specified range. Note: The prescribed minimum and maximum carrier counts also account for the addition of 5% organic soil to the inoculum. a. The mean *TestLD* for carriers inoculated with *M. bovis* (BCG) must be at least 4.0 (corresponding to a geometric mean density of 1.0×10^4) and not above 6.0 (corresponding to a geometric mean density of 1×10^6); a mean *TestLD* below 4.0 and above 6.0 invalidates the test, except for two retesting scenarios (outlined in the study protocol). 3. Management of non-conforming data will be specified in the study protocol; procedures will be consistent with SOP ADM-07, Non-

	Conformance Reports.
9. **Data Management**	Data will be archived consistent with SOP ADM-03, Records and Archives.
10. **Cautions**	1. There are time sensitive steps in this procedure including the use periods of the inoculated carriers and the test chemical. 2. Poor media performance may invalidate the test. 3. A spectrophotometer out of calibration may result in carrier counts outside the range specified in this SOP. 4. To ensure adequate volume of media and diluents, verify volumes in advance and adjust accordingly.
11. **Special Apparatus and Materials**	1. Culture media. a. *Modified Proskauer-Beck medium*. Dissolve 2.5 g KH_2PO_4, 5.0 g asparagine, 0.6 g $MgSO_4 \times 7H_2O$, 2.5 g magnesium citrate, 20.0 mL glycerol, 0.0046 g $FeCl_3$, and 0.001 g $ZnSO_4 \times 7H_2O$ in 1 L H_2O. Adjust to pH 7.2-7.4 with 1 N NaOH. Filter through Whatman No. 4 (or equivalent) filter paper, place 20 mL portions in separate 25 × 150 mm tubes, and steam sterilize 20 min at 121°C. Use this broth for propagating test cultures (25 × 150 mm tubes) and for recovery of test organism from treated carriers (38 × 100 mm tubes). b. *Middlebrook 7H9 agar* (dehydrated M7H9 medium + agar). Dissolve 4.7 g in 900 mL H_2O containing 2 mL glycerol and 15.0 g agar. Heat to boiling to dissolve completely. Steam sterilize 15 min at 121°C. Cool sterile medium to approximately 45°C, add 100 mL Middlebrook ADC Enrichment under aseptic conditions, and mix thoroughly. Distribute in 20 mL portions in sterile 25 × 150 mm screw-capped tubes and slant or dispense a minimum of 30 mL into sterile Petri plates. Use slants to maintain stock culture and plates for inoculum isolation and enumeration. c. *Middlebrook 7H11 agar (dehydrated M7H11 medium)*. Dissolve 21 g dehydrated M7H11 agar medium in 900 mL H_2O containing 5 mL glycerol. Swirl to obtain a smooth suspension; boil if necessary to completely dissolve the powder. Steam sterilize 15 min at 121°C. Cool sterile medium to 50-55°C, add 100 mL OADC enrichment under aseptic conditions, and mix thoroughly. Distribute in 20 mL portions in sterile 25 × 150 mm screw-capped tubes and slant or dispense a minimum of 30 mL into sterile Petri plates. Alternatively, pre-made M7H11 agar plates may be purchased. Use slants to maintain stock culture and plates for

inoculum isolation and enumeration.

d. *Middlebrook 7H9 broth (dehydrated M7H9 medium).* Dissolve 4.7 g in 900 mL H_2O containing 2 mL glycerol and 1.0 g Bacto agar. Heat to boiling to dissolve completely. Steam sterilize 15 min at 121°C. Cool sterile medium to 45°C, add 100 mL Middlebrook ADC Enrichment under aseptic conditions and mix thoroughly. Distribute in 20 mL portions in sterile 25 × 150 mm tubes. Use for recovery of test organism from treated carriers.

e. *Kirchners medium*. Dissolve 5 g asparagine, 2.5 g sodium citrate, 0.6 g magnesium sulfate (heptahydrate), 2.5 g monopotassium phosphate, and 1.5 g dipotassium phosphate, in 900 mL H_2O containing 20 mL glycerol and 1.0 g Bacto agar. Heat to boiling to dissolve completely. Steam sterilize 15 min at 121°C. Cool sterile medium to 45°C, add 100 mL Middlebrook ADC Enrichment under aseptic conditions, and mix thoroughly. Distribute in 20 mL portions in sterile 25 × 150 mm tubes. Use for recovery of test organism from treated carriers.

f. *TB broth base*. Dissolve 2.0 g yeast extract, 2.0 g proteose peptone No. 3, 2.0 g casitone, 1.0 g potassium phosphate monobasic, 2.5 g sodium phosphate dibasic, 1.5 g sodium citrate, and 0.6 g magnesium sulfate (heptahydrate) in 900 mL H_2O containing 50 mL glycerol and 1.0 g Bacto-agar. Heat to boiling to dissolve completely. Steam sterilize 15 min at 121°C. Cool sterile medium to 45°C, add 100 mL Dubos Medium Serum under aseptic conditions, and mix thoroughly. Distribute in 20 mL portions in sterile 25 × 150 mm tubes. Use for recovery of test organism from treated carriers.

2. Test organism.

a. *Mycobacterium bovis* (BCG) (Organon Teknika Corp., Durham, NC, USA, or equivalent). For stock culture, streak inoculate M7H9 or M7H11 agar slants. Incubate 15-20 days at 36 ± 1°C. Following incubation, maintain at 2-5°C for up to 6 weeks.

3. Reagents

a. *Sterile water*. Use reagent-grade water free of substances that interfere with analytical methods. Any method of preparation of reagent-grade water is acceptable provided that the requisite quality can be met. See Standard Methods for the Examination of Water and Wastewater and SOP QC-01, Quality Assurance of Purified Water for details on reagent-grade water.

	b. *0.1% polysorbate 80 in saline*. Add 0.1 mL polysorbate 80 to 100 mL sterile 0.85% aqueous saline (sodium chloride) solution, filter sterilize. Used in test culture preparation. 4. Apparatus. a. *Specialized glassware*. For neutralizer/primary subcultures, use autoclavable 38 × 100 mm tubes (Bellco Glass Inc., Vineland, NJ). Cap tubes with closures before sterilizing. For glassware used to prepare test chemical, refer to SOP MB-22. b. *Tissue grinder*. Kimble glass tissue grinder (catalog number 885300-0015). c. *Inoculating loop*. For culture inoculation, 1 µL sterile disposable loops (Fisher Scientific). For culture harvest, 95% platinum, 3.5% rhodium alloy, 18 or 19 gauge, 4 mm loop with 75 mm shank or equivalent or disposable loops. d. *Carriers*. Glass Slide Carriers, 25 mm × 75 mm (or comparable size) borosilicate glass cover slips with number 4 thickness. Refer to SOP MB-03, Screening of Stainless Steel Cylinders, Porcelain Cylinders and Glass Slide Carriers Used in Disinfectant Efficacy Testing. e. *Sterile surgical gloves*. For handling the towelette. f. *Forceps*. For manipulating glass slides. g. *Micropipettes*. For performing serial dilutions. h. *Positive displacement pipette.* With corresponding sterile tips able to deliver 10 µL. i. *Timer*. Any certified timer that can display time in seconds. j. *Spectrophotometer*. Calibrated; for preparing standardized test culture. k. *Semimicrocuvette with cap.* For measuring percent transmittance. l. *TB Stain Kit.* For presumptive identification of test microbe.
12. **Procedure and Analysis**	An assessment of media quality (performance) is necessary to ensure the validity of the tuberculocidal efficacy results. The media assessment may be conducted in advance of or concurrently with efficacy testing; refer to SOP MB-10, Media and Reagents Used in Microbiological Assays Including Performance Assessment and Sterility Verification. One towelette is used to wipe ten carriers/slides. The area of the towelette used for wiping is folded and rotated so as to expose a new surface of the

	towelette for each carrier. The method may be altered to accommodate various towelette/carrier combinations (e.g., more than one towelette per set of ten slides). The Disinfectant Towelette Test for *M. bovis* (BCG) Processing Sheet (see section 14) must be used for tracking testing activities.
12.1 Test Culture Preparation	Refer to SOP MB-02 for the test microbe culture transfer notation. a. Initiate test culture by inoculating a sufficient number of 25 × 150 mm tubes containing 20 mL MPB (approximately 10) from stock culture slant(s) (M7H9 or M7H11 agar slants) by transferring one to two 1 µL loopfuls from the stock culture onto the surface of the broth. Record all transfers on the Organism Culture Tracking Form (culture notation = –SL, indicating a transfer from slant to liquid). Note: Over-inoculation of MPB may lead to reduced viability due to excessive growth after 21 ± 2 days; the resulting carrier counts may be negatively impacted. b. Incubate the tubes 21 ± 2 days undisturbed at 36 ± 1°C in a slanted position to increase surface area. c. On the test day: Using a transfer loop, transfer culture to a heat-sterilized glass tissue grinder, add 1.0 mL 0.1% polysorbate 80 in saline solution, grind to break up large clumps or aggregates of the test organism. d. Dilute the homogenized culture with 9 mL MPB broth and transfer the suspension from the tissue grinder to a sterile test tube. Harvest and homogenize culture from multiple MPB broth tubes. Note: Growth from multiple tubes may be harvested and combined to prepare the concentrated culture prior to standardization. e. Allow the suspension to settle for 10-15 min. f. Remove the upper portion of each culture, leaving behind any debris or clumps, and transfer to a sterile flask; pool cultures in the flask and swirl to mix. g. Dilute the pooled culture with MPB broth to achieve 20 ± 1% T at 650 nm. Use a semimicrocuvette with cap while measuring transmittance. h. If an organic soil load is specified in the test parameters for the product test, the appropriate amount of organic soil is added to the pooled test culture prior to the inoculation of carriers. Swirl to

	mix. i. Aliquot a sufficient volume of culture into sterile test tubes. j. Use standardized culture to inoculate glass slide carriers.
12.2 Carrier Inoculation	Inoculate approximately 20 carriers; 10 carriers are required for testing, 3 for control carrier counts, and 3 for the viability controls. a. Use a calibrated positive displacement pipette to transfer 10 µL of the test culture onto the sterile test carrier in the Petri dish, at one end of the slide. Do not place inoculum in the middle of the slide. Vortex-mix the inoculum periodically during the inoculation of carriers. Immediately spread the inoculum uniformly over one third of the carrier surface using a sterile loop. Do not allow the inoculum to contact the edge of the glass slide carriers during the inoculation process. Cover dish immediately. b. Dry carriers in incubator at 36 ± 1°C for 30 ± 2 min. Record the timed carrier inoculation activities on the Disinfectant Towelette Test for *M. bovis* (BCG) Processing Sheet (see section 14). Perform efficacy testing within two hours of drying. c. After completion of all slide inoculations, thoroughly wipe the micropipette with 70% ethanol prior to removal from the BSC.
12.3 Enumeration of viable bacteria from carriers (control carrier counts)	a. After inoculated carriers have dried, randomly select 3 inoculated carriers for assay. Assay 1 carrier immediately prior to conducting the efficacy test and 2 carriers following the test. b. Place each of the inoculated, dried carriers in a 38 × 100 mm tube or a sterile 50 mL polypropylene conical tube containing 20 mL of MPB broth and vortex each tube for 15 seconds. Record the time of vortexing on the Disinfectant Towelette Test for *M. bovis* (BCG) Processing Sheet (see section 14). c. Make serial ten-fold dilutions in 9 mL phosphate buffered dilution water. If the serial dilutions are not made and plated immediately, the vortexed tubes are kept at 2-5°C until this step can be done; however, perform dilution and plating within 2 h of vortexing. d. Briefly mix each serial dilution tube prior to plating. Plate 100 µL aliquots of appropriate dilutions in duplicate on M7H9 or M7H11 using spread plating. Dilutions 10^{-1} through 10^{-3} should produce plates with CFU in the appropriate range. Plates must be dry prior to incubation. e. Incubate plates (inverted) concurrently with the efficacy test

	subculture tubes at 36 ± 1°C for 17-21 days. f. Count colonies. Plates that have colony counts over 300 will be reported as TNTC. Record counts on the Disinfectant Towelette Test for *M. bovis* (BCG) Carrier Counts Form (see section 14). See section 13 for data analysis. g. The mean *TestLD* for carriers inoculated with *M. bovis* (BCG) must be at least 4.0 (corresponding to a geometric mean density of 1.0×10^4) and not above 6.0 (corresponding to a geometric mean density of 1×10^6).
12.4 Disinfectant Sample Preparation	a. Prepare disinfectant sample per SOP MB-22. b. Wipe the outside of the towelette packet or dispenser with 70% ethanol and allow to air dry prior to opening.
12.5 Test Procedure	a. Record timed events on the Disinfectant Towelette Test for *M. bovis* (BCG) Time Recording Sheet for Carrier Transfers (see section 14). b. Wipe the outside of the towelette dispenser or packet with 70% ethanol and allow to air dry. c. Aseptically remove several towelettes before aseptically removing a towelette to initiate testing. Fold towelette in half lengthwise one to two times depending on the size. Beginning at the bottom, fold up towards the top five times. The following steps in the "procedure" section are more conveniently done with two analysts – one to manage the Petri dishes and slides, and the other to perform the wiping procedure. d. Remove the lid from the Petri dish and aseptically remove the inoculated slide and hold it firmly against the rim of the Petri dish. e. Wipe the slide back and forth three times lengthwise with the towelette for a total of six passes across the inoculum or as specified by the study sponsor. Wiping should be done within ±5 seconds of specified time. Place slide in Petri dish, close the lid, and allow slide to sit undisturbed for the contact time. Maintain the wiped carriers in a horizontal position. f. Repeat with four additional slides, folding the used section of the towelette in such a way as to expose a new surface for wiping each slide. g. After the fifth slide, unfold the vertical fold in the towelette and reverse the towelette so that the used surface of the towelette faces inward. Continue wiping an additional five slides, folding the

towelette between each slide to expose a new surface.

h. Following the contact time, drain the excess disinfectant from each slide without touching the Petri dish and sequentially transfer into the neutralizer tube within the ±5 second time limit. Perform transfers with sterile forceps. Place the inoculated/wiped end of the slide into the tube.

i. After the slide is deposited, shake tube containing carrier in neutralizer thoroughly; transfer the carrier to the tube containing 20 mL MPB broth within 5-10 minutes. Sterilize forceps after each carrier transfer.

j. Once all carriers have been transferred to the MPB broth tubes, sequentially transfer 2 mL aliquots from each neutralizer tube into duplicate tubes of 2 additional subculture media, M7H9 broth, Kirchners medium, or TB broth, as specified. This portion of the assay is not timed, but the aliquots should be sequentially transferred to the subculture media within approximately 30 ± 5 min. Repeat this with each tube of neutralizer. Shake each subculture tube thoroughly. Slightly loosen caps of growth media prior to incubation.

k. Incubate 60 days at 36 ± 1°C.

l. Report results as + (growth) or 0 (no growth).

m. Record results at 60 days. If the 60^{th} day of incubation falls on a weekend or holiday, record the results on the first workday following the 60^{th} day of incubation.

   i. Tubes may be monitored beginning at day 21 for evidence of typical mycobacterial growth. If multiple tubes show significant growth prior to the 60^{th} day, confirmatory tests (e.g., acid fast staining and streak isolation) may be initiated prior to day 60. If the results of the confirmatory test are indicative of *M. bovis* (BCG), the results may be recorded at that point to expedite the reporting process.

   ii. Provide justification when recording results on days other than 60 in the comments section of the Disinfectant Towelette Test for *M. bovis* (BCG) Results Sheet (see section 14).

n. If no growth or occasional growth (insufficient for confirmatory tests) occurs within a set of tubes, incubate the set an additional 30 days and record the results. Growth should be checked by using

		standard confirmatory procedures (e.g., acid fast staining and growth on M7H9 or M7H11 agar) to ensure that no contamination is present. o. Record results at 90 days. If the 90th day of incubation falls on a weekend or holiday, record the results on the first workday following the 90th day of incubation. Recording of results beyond the 90th day should be notated in the Comments section on the Disinfectant Towelette Test for *M. bovis* (BCG) Results Sheet (see section 14).
12.6	Sterility and viability controls	a. Sterility controls. Place one sterile, uninoculated carrier into a tube of MPB broth. In addition, incubate 1 tube of each subculture medium with 2 mL sterile neutralizer for quality control purposes. Shake each tube thoroughly and incubate all tubes with the efficacy test. Report results as + (growth) or 0 (no growth) as determined by presence or absence of turbidity or presence of culture growth. Growth should not occur in any tube. Record results on the Disinfectant Towelette Test for *M. bovis* (BCG) Results Sheet (see section 14). b. Viability controls. On the day of testing, place a dried inoculated carrier into a tube of MPB broth and a tube of each subculture medium. Incubate tubes as in the efficacy test. Report results as + (growth) or 0 (no growth) as determined by presence or absence of turbidity or presence of culture growth. Growth should occur in all tubes. Record results on the Disinfectant Towelette Test for *M. bovis* (BCG) Results Sheet (see section 14).
12.7	Test microbe identification	a. Presumptively confirm at least one positive subculture tube for each carrier set with growth. The maximum number of tubes subjected to confirmatory tests per disinfectant tested is 10. b. If more than one subculture tube for a carrier set is positive, confirm a minimum of one tube using acid fast staining and isolation on selective media (M7H9 or M7H11 agar plates). c. If the MPB in the set is positive, it is the representative subculture tube used for identification. If MPB is not positive, any of the other subculture media may be used for identification. d. If growth is observed in only one carrier set, then all subculture tubes showing growth for that carrier are subject to confirmatory tests. e. Growth for acid fast staining is taken from the selected positive tubes on the day that results are read. Acid fast rods are typical for

	M. bovis (BCG). The acid fast staining results should be read promptly prior to assigning a + or 0 to the results. If acid fast rods are observed from the selected tubes then a + is assigned to the results. If no cells are observed for the acid fast stain, apply a 0 to the results. f. In addition, streak isolate growth from positive tubes on M7H9 or M7H11 agar and incubate for 17-21 days at 36 ± 1ºC. g. Following the 17-21 day incubation period, evaluate the colony morphology on M7H9 or M7H11 agar. *M. bovis* (BCG) typically appears as colorless to buff-colored, raised, rough growth on M7H9 and M7H11 agar (see Attachment 1). h. If a satisfactory smear cannot be obtained directly from the tube, take the smear for acid fast staining from the 17-21 day old M7H9 or M7H11 agar plate that was inoculated with the growth from the tube. i. In the event that no cells were observed with acid fast staining initially but typical growth was observed on the M7H9 or M7H11, correct the 0 to read + on the test sheet. An entry error will be noted in the comments section of the Disinfectant Towelette Test for *M. bovis* (BCG) Results Sheet (see section 14). j. Record results on the Disinfectant Towelette Test for *M. bovis* (BCG) Microbe Confirmation Sheet (see section 14).
13. **Data Analysis/ Calculations**	Calculations will be computed using a Microsoft Excel spreadsheet (see section 14). Both electronic and hard copies of the spreadsheet will be retained. Counts from 0 through 300 and their associated dilutions will be included in the calculations.
14. **Forms and Data Sheets**	1. Attachment 1: Typical Growth Characteristics of strains of *M. bovis* (BCG) 2. Attachment 2: Culture Initiation and Stock Culture Generation for *Mycobacterium bovis* (BCG) 3. Test Sheets. Test sheets are stored separately from the SOP under the following file names: Physical Screening of Carriers Record — MB-03_F1.docx Organism Culture Tracking Form for *Mycobacterium bovis* (BCG) — MB-07_F6.docx Test Microbe Confirmation Sheet (Quality Control) — MB-07_F7.docx

	Disinfectant Towelette Test for *M. bovis* (BCG) Carrier Counts Form	MB-23-02_F1.docx
	Disinfectant Towelette Test for *M. bovis* (BCG) Time Recording Sheet for Carrier Transfers	MB-23-02_F2.docx
	Disinfectant Towelette Test for *M. bovis* (BCG) Information Sheet	MB-23-02_F3.docx
	Disinfectant Towelette Test for *M. bovis* (BCG) Results Sheet	MB-23-02_F4.docx
	Test Microbe Confirmation Sheet	MB-23-02_F5.docx
	Carrier Count Spreadsheet MS Excel spreadsheet: Carrier Count Template_CTBDTT_v3	MB-23-02_F6.xlsx
	Disinfectant Towelette Test for *M. bovis* (BCG) Processing Sheet	MB-23-02_F7.docx
15. **References**	1. Official Methods of Analysis. Revised 2013. AOAC INTERNATIONAL, Gaithersburg, MD, (Method 961.02). 2. Official Methods of Analysis. 2012. 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, (Method 965.12 In vitro Test for Determining Tuberculocidal Activity). 3. Standard Methods for the Examination of Water and Wastewater. 2005. 21st Ed., American Public Health Association, Washington, D.C. 4. Holt, J., Krieg, N., Sneath, P., Staley, J., and Williams, S. eds. 1994. Bergey's Manual of Determinative Bacteriology, 9th Edition. Williams & Wilkins, Baltimore, MD. 5. Sneath, P., Mair, N., Sharpe, M.E., and Holt, J. eds. 1986. Bergey's Manual of Systematic Bacteriology. Volume 2. Williams & Wilkins, Baltimore, MD. 6. Package Insert – TB Stain Kits and Reagents. Becton, Dickinson and Company. Part no. 8820201JAA. Revision 03/2011.	

Attachment 1

Typical Growth Characteristics of strains of *M. bovis* (BCG) (see ref. 15.4 and 15.5)

	M. bovis (BCG)*
Gram stain reaction	weakly (+)
Acid Fast stain reaction	(+)
Typical Growth Characteristics on Solid Media	
Middlebrook 7H9	rough, raised, thick colonies with a nodular or wrinkled surface and an irregular thin margin, off-white to faint buff, or even yellow
Typical Microscopic Characteristics	
Cell dimensions	0.3-0.6 µm in diameter by 1-4 µm in length*
Cell appearance	rods, straight or slightly curved, occurring singly and in occasional threads

*After 15-20 days

Attachment 2

Culture Initiation and Stock Culture Generation for *Mycobacterium bovis* (BCG)

A1. Culture initiation. Refer to SOP MB-02 for establishment of the organism control number.

a. Obtain lyophilized culture of *M. bovis* (BCG).

b. Reconstitute the lyophilized culture with ~1 mL of sterile DI water. Inoculate two M7H9 or M7H11 agar plates by streaking for isolation.

c. Add ~0.2 mL of the rehydrated culture to each of 4 tubes of MPB.

d. Incubate the M7H9 or M7H11 agar plates and MPB broth tubes for 15 to 20 days at 36 ± 1ºC or until there is sufficient growth. Incubate MPB broth tubes in a slanted position.

A2. Culture maintenance.

a. Confirm the identity of the streak isolation plates and acid fast stain (see Attachment 1 for colony morphology and section 15.5 for acid fast staining). Afterwards, use the 15-20 day old MPB broth cultures (from section A1) to initiate stock cultures.

b. Streak M7H9 or M7H11 agar slants (stock slants) using 1-4 tubes of MPB broth cultures of *M. bovis* (BCG). Based on anticipated use, streak approximately 10-20 stock slants.

c. Incubate the new stock transfers for 15-20 days at 36 ± 1ºC. Store at 2-5ºC.

d. Every 6 weeks (42 days), generate an additional 10-20 M7H9 or M7H11 slants. Inoculate new M7H9 or M7H11 slants by streaking a loopful of *M. bovis* (BCG) growth from an established tube to each of the 10-20 tubes. Perform QC of stock cultures per section A3.

e. Incubate the stock culture slants at 36 ± 1ºC for 15 to 20 days. Following incubation, maintain stock cultures at 2-5ºC for up to 6 weeks.

A3. QC of stock cultures

a. Up to every 6 weeks (42 days), streak a loopful of growth for isolation from the existing M7H9 or M7H11 stock slant used to inoculate new agar slants on a plate of M7H9 or M7H11 agar. Incubate the plate for 17-21 days at 36 ± 1ºC.

b. Following the incubation period, record the colony morphology as observed on the M7H9 or M7H11 plate. See Attachment 1 for details on cell and colony morphology and stain reactions.

c. Perform an acid fast stain (refer to 15.5) from growth taken from the M7H9 or M7H11 streak isolation plate according to the manufacturer's instructions. Observe

the acid fast reaction by using brightfield microscopy at 1000X magnification (oil immersion).

d. Record observations on the Test Microbe Confirmation Sheet (Quality Control) (see section 14).